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Effects of unionized ammonia and suspended solids on rainbow trout (*Oncorhynchus mykiss*) in recirculating aquaculture systems

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22 Abstract

23 This study investigates the individual and combined effects of chronic exposure of rainbow
 24 trout to unionized ammonia and suspended solids in a farm-scale recirculating aquaculture
 25 system (RAS) over 13 weeks. Unionized ammonia nitrogen concentration was four times
 26 (0.05 mg/L) the generally accepted 'safe' threshold while total suspended solids (TSS)
 27 exceeded the 'safe' threshold of 25 mg/L by a factor of > 2.5. Still, rainbow trout revealed
 28 high survival rates of > 99% and no observable detrimental effects of TSS. Bacterial
 29 activity showed a close positive linear correlation with solid load and was almost
 30 exclusively explained by solid load for TSS concentration > 10 mg/L. However, bacterial
 31 activity had no apparent detrimental effect on fish health or performance. Increased
 32 unionized ammonia nitrogen concentrations had no relevant detrimental effect on rainbow
 33 trout physiology and performance at concentrations of up to 0.05 mg/L. Furthermore, the
 34 absent to minor solid-related effects across a wide range of physiological criteria combined
 35 with chronic exposure to unionized ammonia demonstrates that chemical or physical
 36 irritants are not problematic in RAS if other water and holding parameters are optimal.
 37 These findings suggest a greater than expected tolerance of rainbow trout to chronic TSS-
 38 related effects which should result in a revision of water quality threshold criteria for RAS.

39

40 Keywords: Fish health, Water quality, Particle accumulation, Turbidity, Salmonid, Bacterial
 41 activity

42

43 Highlights:

- 44 • Study of combined chronic effects of critical solid and unionized ammonia exposure
- 45 • Full control of water parameters except turbidity in replicated RAS
- 46 • Only minimal effects of $\text{NH}_3\text{-N}$ up to 0.05 mg/L on fish physiology
- 47 • No interaction effects between unionized ammonia and suspended solid load
- 48 • Close linear correlation of suspended solid load and bacterial activity

49

50 1 Introduction

51 Aquaculture is the fastest-growing sector in the animal food production industry worldwide
 52 and already accounts for more than 44 percent of global total fish production (FAO, 2016).
 53 As capture fishery production has remained relatively static since the late 1980s and the
 54 world demand for fish is increasing (FAO, 2016), aquaculture has an important role to play
 55 in ensuring a sufficient global fish supply (Naylor et al., 2000). Recirculating aquaculture
 56 systems (RAS) are often regarded as an environmentally friendly alternative to open flow-
 57 through or cage-based aquaculture systems (Ayer and Tyedmers, 2009; Klinger and
 58 Naylor, 2012; Verdegem et al., 2006), largely due to their efficient water use. However,
 59 despite ongoing development, fish production in RASs remains energy- and cost-intensive
 60 and its contribution to global production is still small (Badiola et al., 2012; Roque
 61 d'Orbcastel et al., 2009). One approach to optimizing the economic output of RASs is to
 62 increase stocking densities to reduce costs per unit of fish produced (Martins et al., 2005).
 63 However, more fish reared in the same volume of water leads to increased excretion loads
 64 per m^3 of water. Fish feces are the principal constituent of suspended solids in
 65 aquacultural facilities along with uneaten feed, bacterial material from biofilters and
 66 microfauna (Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al.,
 67 1999; Wedemeyer, 1996). Accumulating particles, and especially fine particles, are
 68 considered detrimental to fish health, welfare and performance (Bilotta and Brazier, 2008;

Chapman et al., 1987; Chen et al., 1993; Chen and Malone, 1991; Herbert and Merkens, 1961). However, this assertion has been questioned for rainbow trout by recent investigations (Becke et al., 2017, 2018). Nevertheless, intensification of aquacultural production resulting in an increase in suspended solid concentration, will also lead to an increase in dissolved wastes, such as unionized ammonia (NH_3) (Ip et al., 2001). High unionized ammonia levels have a wide range of detrimental effects on fish, e.g. deterioration of gill structures, and might ultimately lead to mortality (Cameron and Heisler, 1983; Daoust and Ferguson, 1984; Ip et al., 2001; Randall and Tsui, 2002; Smart, 1976; Thurston et al., 1984; Wicks et al., 2002). The common upper safe limit of unionized ammonia-N proposed for salmonid aquaculture is 0.0125 mg/L (Timmons and Ebeling, 2010). However, there are studies reporting higher tolerance (Daoust and Ferguson, 1984; Meade, 1985). Thus, there is still controversy about the safe threshold for unionized ammonia in aquaculture operations.

A recent factor significantly influencing water quality in aquaculture is a change in feed composition. Fish meal and fish oil are increasingly being substituted by plant alternatives in salmonid diets (Glencross et al., 2007; Ytrestøyl et al., 2015). This is partly due to declining fish stocks and rising prices for fish meal and fish oil (Naylor et al., 2009). This replacement coincidentally causes a less dense and more fragile composition of fish feces (Schumann et al., 2018; Unger and Brinker, 2013), considerably increasing fine suspended solids in fish farm waters (Brinker and Friedrich, 2012).

Against this background, the present study investigated the sole effect of critical unionized ammonia-N concentrations (> 0.0125 mg/L) as well as interaction effects with suspended solid load in a farm-scale RAS. It was hypothesized that chronic exposure to high unionized ammonia concentrations would cause a reduction of fish wellbeing, while the combined chronic exposure with increased suspended solid load would provoke an interactive impact. Within this context, husbandry waters were set to optimal values except

for the two variables, unionized ammonia and suspended solids, being tested. The exception was bacterial activity which was held at an uncritical level (Pedersen et al., 2017; Rojas-Tirado et al., 2018), with possible covariate influences being controlled by the experimental design.

2 Materials and Methods

2.1. Husbandry

The experiment used two replicate RASs, each with 10 tanks (capacity of 330 L, total RAS volume 6m³) (Figure 1), as described by Becke et al. (2018). The study used all-female rainbow trout (*Oncorhynchus mykiss*, Störk strain) to exclude sex-related effects. Each RAS was stocked with 785 rainbow trout with an average initial weight of 87.2 ± 8.6 g (control group) and 87.4 ± 9.2 g (treatment group). They were held at maximum stocking densities of 67.8 ± 3.0 kg m⁻³ (control) and 68.3 ± 2.6 kg m⁻³ (treatment). The control RAS was operated under regular conditions, while the particle load of the treatment RAS was artificially elevated as described in Becke et al. (2018). Briefly, a mud pump (Wilo-EMU KS 8 ES, Dortmund, Germany) was used to pump the backwash water of the drum filter back into the system. In both systems, the drum filter (HDF801-1H, Hydrotech, Vellinge, Sweden) was equipped with a 100 µm gauze, so that particles < 100 µm accumulate over time.

The photoperiod was fixed at 12L:12D with a sigmoidal transition period of 30 min (Lumilux daylight lamps) with different light intensities of 50, 100, 200, 300 and 600 lx in duplicate per system. However, without any significant effect on the results presented (unpublished data). The fish were fed restrictively according to supplier recommendations with a commercial feed (Biomar EFICO Enviro 921, Aarhus C, Denmark), by hand six days a week (Sunday to Friday) at 2.5 % of body weight at the beginning of the trial, declining to 1.3 % by the end (maximum feed amount was 2.84 kg/day per RAS). Bacterial growth was

121 controlled with UV irradiation of the system water (Barrier L20, Wallace & Tiernan,
122 Günzburg, Germany; UV dose: 40 mJ/cm² flow volume: 6600 L/h, lamp wattage: 80 W,
123 measurement range UV sensor: 200W/m²). Fish (average weight approx. 15 g) were put
124 into the two RASs three months before the beginning of the experiment to ensure
125 acclimatization.

126

127 2.2. Water parameters

128 The experiment was subdivided into three phases (Figure 2): in phase 1 (week 1 – 5),
129 water parameters in both RASs were kept at levels known to preclude negative impacts on
130 fish health or performance (Table 1). In the treatment RAS, however, the total suspended
131 solid concentration was increased to over 35 mg/L and was subsequently held constantly
132 above this value. The control RAS operated under commercial conditions at around 5
133 mg/L throughout the experiment. In phase 2 (week 6 – 10), ammonium concentration was
134 artificially elevated in both RASs by adding ammonium chloride (A7012,9025; AppliChem,
135 Darmstadt, Germany). Additionally, biofilter efficiency in both RASs was reduced by
136 halving the volume of carrier material (originally designed for 4.5 kg feed/day) to attain
137 higher NH₄-N concentrations. Ammonium nitrogen concentration was measured in both
138 RASs every 60 minutes using an automat (AMTAX SC, Hach, Germany). In addition, pH
139 was increased from 7.5 to around 8 in both RASs to increase the proportion of **unionized**
140 **ammonia nitrogen** (NH₃-N) to approximately 0.0125 mg/L (Figure 3). The increase in pH
141 was achieved by adding sodium hydrogen carbonate, dissolved in water, using a peristaltic
142 pump (Concept 420i, Saier Dosiertechnik, Germany). The pH was constantly monitored
143 using OxyGuard pH-probes (Farum, Denmark). **The concentration of unionized ammonia-**
144 **N was calculated based on actual pH and temperature according to Emerson et al. (1975).**
145 In phase 3 (week 11 – 13), the concentration of **unionized ammonia-N** was further
146 increased to an average of approximately 0.025 mg/L (Figure 3).

147 $\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ were chemically determined three times per week throughout
 148 the experiment with analysis kits (LCK 304: 0.2 – 2.5 mg/L; LCK 341: 0,05 – 2 mg/L; and
 149 LCK 339: 1 – 6 mg/L, Hach, Germany, respectively), using water from the connecting tube
 150 from the fish tanks of each RAS. Oxygen concentration (using Oxygen Probes, OxyGuard,
 151 Farum, Denmark) and temperature (using Temperature Probes, Oxyguard, Farum,
 152 Denmark) were monitored continuously at the outlets of two fish tanks in each system.
 153 Carbon dioxide concentrations were determined two times per week in the fish tanks using
 154 a portable dissolved CO_2 analyzer (OxyGuard CO_2 Portable, OxyGuard, Farum,
 155 Denmark). Turbidity was determined three times per week in parallel with the
 156 determination of total suspended solids using a turbidity meter (PCE-TUM 20, PCE
 157 Instruments, Germany).

159 2.3. Analysis of suspended solids

160 2.3.1. Total suspended solids

161 The concentration of total suspended solids was determined three times per week in
 162 duplicate for each system according to method 2540 D of the American Public Health
 163 Association (APHA, 1998), with the exception that 0.45 μm cellulose-acetate filters
 164 (diameter: 50mm, 11106-50-N, Sartorius AG, Göttingen, Germany) were used instead of
 165 glass-fiber filters due to the smaller and better defined pore sizes. Filters were prepared as
 166 described by Becke et al. (2018). Water samples were collected using a tube at a water
 167 depth of ca. 30 cm from five tanks in each system, then duplicate samples were pooled to
 168 create a representative sample for each system. Samples were collected in the early
 169 morning before feeding, in order to represent the daily minimum solid loads (best case
 170 scenario). To determine the within-day fluctuations and maximum values, measurements
 171 were performed every two hours on one day in week 12.

172

173 2.3.2. Particle size distribution (PSD)

174 For particle size measurement, water samples were collected as described above. Particle
175 sizes were determined according to Brinker et al. (2005) using a non-invasive laser particle
176 sizer (GALAI:CIS-1, GALAI Productions Ltd. Midgal Haemak, Israel) equipped with a flow
177 controller (GALAI:LFC- 100) and a flowthrough cell (GALAI:GM-7). The measurements
178 were performed in quadruplicate for each system in week 12 of experimental operation.

179

180 2.4. Fish performance

181 The specific growth rate (SGR) was calculated from mean weights recorded at the
182 beginning and the end of the experiment by using the following formula:

$$183 \text{ SGR } (\%d^{-1}) = (\ln(\text{mean final weight}) - \ln(\text{mean initial weight})) / (t(\text{final day}) - t(\text{initial day})) \times 100;$$

184 where t is time (days).

185 The feed conversion ratio (FCR) was calculated as:

$$186 \text{ FCR} = \text{Feed (kg)} / \text{Weight gain (kg)}$$

187 The thermal growth coefficient (TGC) was calculated according to Jobling (2003):

$$188 \text{ TGC} = (W_t^{1/3} - W_0^{1/3}) \times (\sum T)^{-1} \times 1000;$$

189 where W_t and W_0 are the final and initial weights (g), respectively and $\sum T$ is sum day-
190 degrees Celsius (Cho, 1992; Iwama and Tautz, 1981).

191

192 2.5. Sampling protocol

193 Fish were sampled at the beginning, in week 5, in week 10 and in week 13 of the study.

194 Fish were fasted 24 h prior to each sampling. Two fish from each tank per system ($n = 20$)
195 were caught and anaesthetized using clove oil (concentration: 0.1 mL/L, exposure time:
196 ca. 60 s). Directly following anesthesia, wet weight (to the nearest 0.1 g) and total length

197 (measured from the tip of the mouth to the end of the tail fin; to the nearest 0.1 cm) of each

198 fish were measured and blood samples were taken from the caudal blood vessels and

199 transferred to tubes containing lithium heparin (25 IU/mL blood, Sarstedt, Nümbrecht,
200 Germany). Subsequently, fish were killed and samples of gill tissue were collected for
201 histological examination.

202

203 2.6. Health parameters

204 2.6.1. Gill histology

205 Gill tissue was prepared and examined as described by Becke et al. (2018). Briefly,
206 observed changes were ranked rising in pathology from 0 (no change) to 3 (severe
207 change) including sub-steps 1 (minor change) and 2 (moderate change). For each section,
208 5 images showing 6–7 secondary gill lamellae were inspected at a magnification of 200×
209 using a photomicroscope (Zeiss, Oberkochen, Germany). Branchial epithelium thickness
210 (µm) was measured at 10 locations in each image and a mean value was calculated. The
211 number of goblet cells was counted per secondary lamella. The gills of 20 rainbow trout
212 from each RAS were investigated at each sampling point.

213

214 2.6.2. Fin condition

215 Fin erosion as an indicator of fish welfare was assessed according to Person-Le Ruyet et
216 al. (2007), and the fin index was determined according to Kindschi (1987), as follows:

$$217 \text{ Fin index} = (\text{fin length} / \text{total length}) * 100$$

218

219 2.6.3. Hematology

220 Hematological parameters (differential leukocyte count, hematocrit, leukocrit, hemoglobin
221 concentration, total red and white blood cell counts) were determined as described by
222 Becke et al. (2018). Glucose concentration was determined using a common glucose
223 measuring device (ACCU-CHEK Aviva, Roche, Mannheim, Germany) as it has been

224 shown that devices for measuring human glucose level are also suitable for use with fish
225 blood (Bartoňková et al., 2017; Eames et al., 2010).

226

227 2.7. Bacterial assay

228 2.7.1. Bacterial load

229 Analysis of bacterial load of rainbow trout was conducted at the termination of the study
230 (20 rainbow trout per RAS) by the fish health service at a governmental veterinary institute,
231 the Staatliches Tierärztliches Untersuchungsamt (STUA) Aulendorf, Germany as
232 described in Becke et al. (2018). Briefly, the number of colony forming units was assessed
233 on skin and spleen and **ranked** as *no*, *sporadic*, *slight*, *moderate* or *severe* bacterial load.
234 Bacterial species were then determined by using bacteriological standard methods and
235 confirmed by MALDI-TOF MS (Lay, 2001).

236

237 2.7.2. Bacterial activity in the water

238 Bacterial activity in the fish tanks was assessed using a patented method called
239 BactiQuant® (Mycometer A/S, Copenhagen, Denmark), which is an indirect measure of
240 microbial enzyme activity. Reproducibility and repeatability of the method has been
241 documented in a verification report by the United States Environmental Protection Agency
242 (U.S.-EPA, 2011). Briefly, a 10 mL water sample was filtered through a Millipore 0.22 µm
243 closed filter unit (PES express). The filter was then incubated with a fluorogenic enzyme
244 substrate for 15 min. The synthetic fluorescent enzyme-substrate is hydrolyzed by
245 microbial enzymes in the water sample and the amount of released fluorophores was
246 quantified with a fluorometer (Mycometer A/S, Copenhagen, Denmark). The results were
247 expressed in standardized Bactiquant® values (BQV; hereafter termed bacterial activity).
248 Measurements were always performed in duplicate. During the first three weeks, bacterial
249 activity was measured every second day to gain a better overview of the development until

250 the particle concentration exceeded 35 mg/L in the treatment RAS. From week 4 onwards,
251 bacterial activity was measured twice a week.

252

253 2.8. Data analysis

254 Data were checked for homoscedasticity using Levene's test (Levene, 1960) and for
255 normality using normal quantile plots. If normal distribution and homoscedasticity tests
256 were passed, treatment effects were tested by *t*-tests, otherwise Wilcoxon tests were
257 employed (Sokal and Rohlf, 2003). For analysis of bacterial activity, branchial epithelium
258 thickness and number of goblet cells per secondary lamella a linear parametric model was
259 applied (Supplement 1).

260 Fin erosion and gill histology parameters (thickening of epithelial cells, cellular edema, cell
261 infiltration, tip thickening, detachment of the epithelium, telangiectasia and lamellar fusion)
262 were tested using a logistic regression on ordinal data. The method of least squares was
263 used to analyze the relation between TSS concentration and turbidity. Bacterial load data
264 of the gills was analyzed using Fisher's exact test. A generalized linear model (GLM) was
265 used to analyze fin index and hematological parameters.

266 The coefficient of variation (C_v) as a unit for the relative standard deviation was calculated
267 in terms of bacterial activity as follows:

$$268 \quad C_v (\%) = (\text{standard deviation } (\sigma) / \text{arithmetic mean } (\bar{x})) \times 100$$

269 All data analyses were performed with JMP Pro (SAS Institute Inc.) version 13.2.1. (64-bit)
270 Differences between treatment groups were considered to be significant at $P < 0.05$.

271

272 3 Results

273 3.1. Water parameters

274 Water temperature differed significantly ($P < 0.001$) between control and treatment RAS,
275 although the absolute difference was small and below 0.6 °C (Table 1). In week 11 to 13,

NO₂-N concentration was approximately 0.15 mg/L higher in the control RAS and differed significantly ($P < 0.05$) between RAS systems, but not in phase one and two of the experiment ($P > 0.05$). Water consumption was significantly higher ($P < 0.05$) in the control system in week 1 to 5 (approx. 40 L/day) and in week 6 to 10 (approx. 22 L/day) than in the treatment because the backwash water of the drum filter was reinjected into the treatment system. From week 11 to 13 no significant difference ($P > 0.05$) was found between systems due to adjusting the water consumption in the treatment system. However, magnitudes of differences were minimal and were thus deemed biologically not relevant. Turbidity differed significantly ($P < 0.001$) by up to 15 NTU (Nephelometric Turbidity Units) between control and treatment RAS at individual sampling time points (Table 1) as related to different suspended solid load. NH₄-N concentrations, pH, O₂ concentration and NO₃-N concentration did not differ significantly ($P > 0.05$) between control and treatment system. NH₄-N concentrations were increased both in the control and treatment RAS after week 5 and week 10 with concentrations peaks of up to 2.5 mg/L (control) and 2.3 mg/L (treatment) respectively, but without significant differences ($P > 0.05$) between systems. Unionized ammonia-N concentrations were also increased after week 5 from 0.005 to 0.012 mg/L and further to over 0.02 mg/L after week 10 (Figure 3), however, without significant differences ($P > 0.05$). Overall, with the exception of NH₄-N/NH₃-N and suspended solid load, all water parameters remained within physiological optimal range for rainbow trout (Timmons and Ebeling 2010).

3.2. Suspended solids analysis

3.2.1 Total suspended solids

Total suspended solid (TSS) concentration differed significantly ($P < 0.0001$) between control and treatment RAS with an average concentration of 4.5 mg/L in the control and 35.2 mg/L in the treatment system (Figure 4 A). From week 3, the TSS concentration in

the treatment system exceeded 30 mg/L and remained at an average of 40.5 mg/L. Furthermore, the difference in TSS concentration between control and treatment RAS was never less than 23.1 mg/L. Figure 4 B shows the within-day variation of the total suspended solids concentration in the control and treatment RAS in week 12 of the experiment with minimum values in the morning at 7:00 a.m. The highest TSS concentration on that day was 65.8 mg/L in the treatment RAS while it was 14.1 mg/L in the control RAS.

3.2.2. Particle size distribution

At week 12, the total number of particles per liter in the treatment RAS was on average more than double that of the control. The average suspended particle load was 17.1 ± 2.1 mg dry weight/L in the control and 47.4 ± 2.7 mg dry weight/L in the treatment system. For each particle size class, the absolute frequencies differed significantly ($P < 0.001$) between control and treatment RAS (Figure 5). Overall, a high accumulation of fine particles occurred in both the control and the treatment RAS, with 98.6 % and 98.3 % of all particles respectively smaller than 15 μm , however, with higher quantities in the treatment RAS.

3.3. Fish performance

In contrast to the expectations based on recommended threshold values, fish performed very well in both systems. A slight difference in feeding behavior was observed between fish in the two systems with a less aggressive and calmer feeding behavior in the treatment RAS. Overall, no significant differences ($P > 0.05$) were apparent for final weight, survival rate, FCR, SGR and TGC between rainbow trout of the control and treatment RAS (Table 2).

3.4. Health parameters

3.4.1. Gill histology

No severe histological changes in gill structures were observed during the investigation. Cases of cellular edema, tip-thickening of secondary lamellae, telangiectasia, thickening of epithelial cells, cell infiltration, lamellar fusion, merging of secondary lamellae and detachment of the epithelium were only minor or moderate (Supplement 2). In terms of cellular edema, all factors were significantly altered by treatment ($P < 0.05$), but magnitude of differences was small (0 – 15 %) and the observed histological change was only rated as minor. The increased TSS concentration did not have any significant effect on all further investigated histological parameters ($P > 0.05$). However, the increased unionized ammonia concentration ($P < 0.05$) and the interaction of unionized ammonia concentration and day of sampling ($P < 0.05$) led to a significantly more frequent occurrence of cell infiltrations and tip thickening of secondary lamellae. All other histological parameters were not significantly affected by the increased unionized ammonia concentration ($P > 0.05$). Furthermore, no significant interaction of increased unionized ammonia concentration and increased suspended solid load ($P > 0.05$) were found for any of the investigated histological parameters. Regarding thickness of branchial epithelium and number of goblet cells per secondary lamella (Supplement 3), no significant effects ($P > 0.05$) of increased unionized ammonia or suspended solid load were apparent at all.

3.4.2. Fin condition

Neither total suspended solid concentration ($P > 0.05$) or unionized ammonia ($P > 0.05$) had a significant effect on fish welfare measured by fin erosion in the control and treatment RAS (Supplement 4). Furthermore, no interaction effect of increased unionized ammonia concentrations and suspended solid load was apparent ($P > 0.05$).

353 The increased **unionized ammonia** concentrations caused a significantly lower fin index
 354 ($P < 0.05$) for the dorsal fin (Supplement 5). However, fin indices of the left and right
 355 pectoral fin were not affected ($P > 0.05$). Increased suspended solid load had no
 356 significant effect ($P > 0.05$) on any fin index. Furthermore, the elevated **unionized**
 357 **ammonia** concentrations did not significantly affect the impact of suspended solid load
 358 ($P > 0.05$) on fin indices.

359

360 3.4.3. Hematology

361 Overall, all hematological parameters (Table 3) were approximately within the range
 362 previously reported for salmonids (McCarthy et al., 1973, 1975; Pund, 1998; Řehulka et
 363 al., 2004). However, hematocrit was significantly decreased ($P < 0.05$) both with
 364 increasing TSS concentration and increasing body length, whereas hematocrit significantly
 365 increased ($P < 0.05$) over time. Thus, the MCV value was also significantly lower ($P <$
 366 0.01) and the MCHC value significantly higher ($P < 0.01$) with increasing TSS
 367 concentrations. The interaction of TSS concentration and **unionized ammonia**
 368 concentration revealed a significant effect ($P < 0.05$) on MCHC values. The number of
 369 thrombocytes was significantly elevated ($P < 0.05$) with increasing **unionized ammonia**
 370 concentration and the hemoglobin concentration significantly increased ($P < 0.05$) over
 371 time. All the other parameters (glucose concentration, number of erythrocytes, MCH,
 372 number of leukocytes, leukocrit and the proportions of lymphocytes, granulocytes and
 373 monocytes) were not significantly affected ($P > 0.05$) by suspended solid load, **unionized**
 374 **ammonia** concentration or the interaction of both parameters.

375

376 3.5. Bacterial assay

377 3.5.1. Bacterial load

Overall, no critical bacterial load was detected in the control or treatment RAS. Bacterial load of the gills differed significantly between fish of the control and treatment RAS in terms of direct detection ($P < 0.01$) with 45 % and 10 % of the fish gills showing no bacterial load in the control and treatment RAS respectively (Figure 6). In contrast, 90 % of the fish gills in the treatment RAS and only 40 % of the fish gills in the control RAS revealed slight to moderate bacterial load. However, no significant difference appeared in terms of cultivation ($P > 0.05$). The bacterial load of the spleen was significantly higher ($P < 0.0001$) for rainbow trout in the suspended solids enriched RAS. In the control RAS, 95 % of the spleens revealed no to sporadic bacterial load, whereas in the treatment system 75 % of the spleens revealed slight to moderate bacterial load. The examination of the skin revealed no bacteria or ectoparasites in either RAS. The fish pathogenic bacteria *Flavobacterium columnare* was detected on two rainbow trout from the control RAS and on four rainbow trout from the treatment RAS by direct detection. The cultivation of gill smears proved the occurrence of *Aeromonas sobria* for three fish in the treatment RAS, but not in the control RAS.

3.5.2. Bacterial activity

Bacterial activity ranged between 0.12×10^5 and 0.47×10^5 in the control RAS ($C_v = 18.0$ %) and between 0.33×10^5 and 3.42×10^5 in the treatment RAS ($C_v = 18.3$ %) (Figure 7). Bacterial activity was only significantly affected ($P < 0.0001$) by the total suspended solid concentration. With increasing particle load in the treatment RAS, bacterial activity increased from about 0.3×10^5 to over 2.6×10^5 during the first three weeks. In contrast, bacterial activity in the control RAS remained roughly static between 0.2×10^5 and 0.3×10^5 during this time. Unionized ammonia-N concentration had no significant effect ($P > 0.05$) on bacterial activity in either RAS. Bacterial activity measured on one representative day in week 8 showed diurnal variations from 0.2×10^5 to 0.4×10^5 in the control RAS and from

2.3 x10⁵ to 3.7 x10⁵ in the treatment RAS respectively. Overall, there was a significant positive linear correlation between TSS concentration and bacterial activity ($P < 0.0001$, $r^2 = 0.98$; Figure 8). However, the certainty measure of the linear correlation of bacterial activity with TSS was very low in the control RAS ($r^2 = 0.10$) while it was high in the treatment RAS ($r^2 = 0.94$).

4 Discussion

The experiment effectively decoupled the effects of chronic suspended solid load and elevated unionized ammonia concentrations from other relevant water quality parameters. This allowed an investigation of the sole effects of both increased unionized ammonia concentrations and suspended solid load on rainbow trout as well as their combined effects at a farm-scale.

Recent investigations (Becke et al., 2017, 2018) have shown that even massive accumulation of fine solids alone caused no detrimental effects on rainbow trout in RAS. These results were corroborated by the present findings which did not reveal relevant detrimental effects of increased suspended solid concentrations on fish at concentrations of up to almost 70 mg/L. Gills are of delicate structure and therefore highly sensitive to physical impact (Evans, 2005; Morgan and Tovell, 1973), so the absence of any histological alteration associated with suspended solid load is of particular note. This is in line with Goldes et al. (1988) who also observed no branchial pathology in rainbow trout even when exposed to up to 1017 mg/L of suspended clay kaolin. Thus, the assumption that suspended solids alone are not a key issue affecting fish welfare in RAS is further strengthened.

However, the increased particle load caused indirect effects. It led to increased turbidity which suppressed feeding behavior of fish in the treatment RAS as previously described (Barrett et al., 1992; Becke et al., 2017, 2018; Utne-Palm, 2002). This altered feed uptake

430 can potentially lead to a loss of feed in commercial settings using automatic feeders. To
 431 preclude this potentially disturbing effect, fish in this study were hand fed which secured
 432 the uptake of all feed pellets.

433 Furthermore, the increased suspended solid load induced a substantial increased bacterial
 434 load. Such a finding was expected (Becke et al., 2018) as an increased number of
 435 particles in the treatment RAS promotes bacterial growth by providing a larger surface
 436 area for bacterial colonization and food-substrate. Bacterial activity levels found in this
 437 study have been observed in other recent studies rearing rainbow trout in intensive RAS
 438 (Pedersen et al., 2017; Rojas-Tirado et al., 2018). Especially remarkable is the close linear
 439 correlation between bacterial activity and TSS, which is however quite variable at low TSS
 440 (< 5 mg/L), but nearly exclusively determined by TSS at high TSS loads. This novel
 441 outcome is of high relevance for systems with need for bacterial control. However, the
 442 physiological parameters investigated here did not reveal any evidence for bacterially
 443 mediated physiological stress response in the control or in the treatment systems. This
 444 was confirmed by the independent veterinary inspection of the rainbow trout which did not
 445 reveal any relevant pathological bacterial infestation. In contrast, Redding et al. (1987)
 446 observed a reduced tolerance to subsequent infection with *Vibrio anguillarum* for yearling
 447 steelhead when exposed to high concentrations of suspended topsoil. In the present
 448 study, however, no bacterial diseases occurred despite very high bacterial and suspended
 449 solid load in the treatment RAS. However, under different conditions, the interaction of
 450 suspended solids and bacterial occurrence might impair fish health and need to be
 451 controlled (Herbert and Merkens, 1961; Qualls et al., 1983).

452 Increased particle concentrations, e.g. due to increased stocking densities in RAS, are
 453 often accompanied by a decrease in water quality because of leaching of harmful
 454 substances or particle-mediated growth of heterotrophic bacteria (Chen et al., 2003; Ling
 455 and Chen, 2005). To simulate this phenomenon on a farm-scale, the concentration of

unionized ammonia-N was increased to levels which exceeded the common upper safe limit of 0.0125 mg/L proposed for salmonid aquaculture (Timmons and Ebeling, 2010). It was hypothesized that the chronic exposure to increased unionized ammonia concentrations would result in a deterioration of physiology and performance of rainbow trout. However, contrary to the hypotheses and praxis as well as academic opinion (Smith and Piper, 1975; Thurston et al., 1984; Timmons and Ebeling, 2010), rainbow trout exposed to chronic unionized ammonia-N concentrations of more than four times the critical threshold did not reveal deteriorated performance in our study. Fish in both systems showed very good performance with nearly 100 % survival. Only minor physiological effects of increased unionized ammonia concentration on gill structure were observed. Nonetheless, the observed alterations of gill structures were only slight to moderate and only two (cell infiltrations, tip thickening of secondary lamellae) out of seven parameters were significantly affected by the increased unionized ammonia load. Thus, these results suggest that the rainbow trout can cope well with the given unionized ammonia concentrations. The common upper safe limit of unionized ammonia-N of 0.0125 mg/L proposed for salmonid aquaculture is based on the findings of Smith and Piper (1975). However, other authors, such as Meade (1985), Daoust and Ferguson (1984) (laboratory experiment) and Kolarevic et al. (2013) (commercial scale) previously questioned the proposed unionized ammonia limit. Nevertheless, the value of 0.0125 mg/L has been echoed widely since then and established in aquaculture textbooks (e.g. Timmons and Ebeling, 2010). However, it has to be noted that oxygen concentration was low (around 6 mg/L) in the Smith and Piper (1975) study. According to Lloyd (1961) and Brown (1968), unionized ammonia toxicity increases with decreasing oxygen levels. Thus, the interaction of low oxygen with high unionized ammonia concentration in the study of Smith and Piper (1975) might be causative for the pathological changes in the gills of rainbow trout. During the present study, however, the system water was saturated with oxygen during the whole

482 investigation period. Thus, in relation to oxygen, **unionized ammonia** toxicity was kept to a
483 minimum which might explain the observed low impact. Overall, the presumption for a
484 higher tolerance level of rainbow trout to **unionized ammonia** was confirmed by the results
485 here showing no relevant effects on fish physiology at the given **unionized ammonia**
486 concentrations.

487 In this context, the stress-modulated effects are important given that stressed fish are
488 more vulnerable to external **unionized ammonia toxicity** than unstressed fish (Randall and
489 Tsui, 2002). Thus, the low impact of elevated **unionized ammonia** concentrations while
490 concomitantly exposed to high fine particle loads render the solid exposure harmless as
491 well.

492 Regarding the impact of **unionized ammonia** on fin condition, only the dorsal fin was
493 negatively affected. As fin condition is frequently consulted to assess fish welfare (Ellis,
494 2002; Ellis et al., 2008; Turnbull et al., 2005), the almost complete absence of any fin
495 deterioration here is remarkable and indicates the very low impact of the **unionized**
496 **ammonia** and solid stressors. Abbott and Dill (1985) assumed that aggressive interaction
497 is the major cause of fin damage in hatchery salmonids. In the present study, fin condition
498 of rainbow trout was marginally better in the solid enriched RAS than in the control RAS.
499 This might be attributable to the calmer behavior and reduced social interaction of fish due
500 to the turbid conditions in the treatment RAS (Bash et al., 2001).

501 The analysis of hematological parameters revealed significant effects for individual
502 parameters both in terms of suspended solids and **unionized ammonia**. However, taking all
503 hematological parameters together, there was no indication of pathological effects. Knoph
504 and Thorud (1996) also did not observe any negative effect of **unionized ammonia-N** up to
505 0.112 mg/L on hematological parameters (hematocrit, RBC count) of Atlantic salmon.
506 Furthermore, Becke et al. (2017, 2018) observed no significant effects of suspended solid
507 load up to 70 mg/L on hematological parameters.

As a consequence of the massive accumulation of fine particles in the treatment RAS and the additionally increased **unionized ammonia** concentration in both systems, it was hypothesized that a multiplicative effect of these two parameters would occur in the treatment RAS, resulting in significant consequences on trout physiology. However, none of the investigated physiology parameters revealed any relevant multiplicative effects of particle and **unionized ammonia** load. **In contrast to our hypothesis, no synergistic impact of increased unionized ammonia concentrations and suspended solid load on fish physiology was found.** These results indicate that the commonly used upper safe limits of 0.0125 mg/L for **unionized ammonia-N** and 25 mg/L for total suspended solids do not represent the actual critical limits for salmonid aquaculture. Fish have evolved mechanisms to counteract high **unionized ammonia** environments, as shown for rainbow trout (Randall and Tsui, 2002; Wicks and Randall, 2002). This suggests that rainbow trout have probably developed an improved tolerance to poor water quality in the course of artificial selection for aquaculture. Positive effects of moderately elevated ammonia concentrations have even been observed for rainbow trout when fed to satiation (Linton et al., 1997, 1999; Wood, 2004). Thus, more research that keeps track of breeding developments is needed to clarify the exact effects of water parameters on rainbow trout and fish in general both in aquacultural production and natural conditions. It might be that the upper safe limits of certain water quality parameters currently used in aquacultural production no longer correspond to the present genetic makeup of fish and that they should be revised.

529

530 5 Conclusions

The results from this study provide a fully controlled insight into the combined effects of particle accumulation and **unionized ammonia** load on physiology of rainbow trout in RAS on a farm-scale. Against expectations and widespread opinion, the solid fraction of the

534 experimental system, comprising almost exclusively fine particles at concentrations
535 distinctly above values normally reached in aquacultural production, failed to provoke
536 detrimental effects on physiology and performance of rainbow trout. The same holds for
537 unionized ammonia and the combination of both.

538 The results therefore indicate with respect to suspended solids and unionized ammonia
539 that increasing fish densities to improve the economic performance of RAS beyond current
540 limits of suspended solids and unionized ammonia is feasible, if accompanying water
541 parameters are optimal.

542
543 Thus, the main conclusions are:

- 544 • Bacterial activity was strongly affected by increased TSS concentrations, but
545 without detrimental effects on fish physiology
- 546 • increased unionized ammonia-N concentrations up to 0.05 mg/L caused only minor
547 effects on fish physiology
- 548 • no relevant combined effects of increased unionized ammonia-N concentrations
549 and suspended solid load were observed
- 550 • upper safe limits of unionized ammonia-N and suspended solids need to be revised
551 for salmonid aquaculture

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- 758
- 759

760 Figure captions:

761

762 Figure 1: Scheme of the recirculating aquaculture systems with modification for particle
763 accumulation in the treatment system (light grey shaded).

764

765 Figure 2: Experimental setup of the rainbow trout exposure in the RAS.

766

767 Figure 3: Unionized ammonia nitrogen ($\text{NH}_3\text{-N}$) concentration (mean, minimum (Min) and
768 maximum values (Max)) in the control and treatment RAS during the investigation period.
769 The dashed line shows the common limit value of $\text{NH}_3\text{-N}$ (0.0125 mg/L) for salmonids
770 (Timmons and Ebeling, 2010).

771

772 Figure 4: (A) Timeline of total suspended solids concentration (mean \pm S.D.) in control and
773 treatment RAS over the experimental period. (B) Representative daily variation of total
774 suspended solids concentration (mean \pm S.D.) in control and treatment RAS in week 12.
775 Samples were collected every two hours between 7:00 and 19:00 and at 23:00 (CET).
776 Please note the axis break on the x-axis.

777

778 Figure 5: Absolute frequency within particle size classes (mean \pm S.E.) of the control (n =
779 4) and treatment RAS (n = 4) in week 12. All particle size classes differed significantly ($P <$
780 0.001) between control and treatment system. Please note the axis break on the y-axis.

781

782 Figure 6: Bacteriological examination of gills (direct detection and cultivation) and spleen
783 (cultivation) from 20 rainbow trout of the control (C) and treatment (T) RAS. ** = $P < 0.01$;
784 *** = $P < 0.0001$

785

786 Figure 7: Bacterial activity (BQV/mL, mean \pm S.D.) in the treatment and control RAS during
787 the investigation period.

788

789 Figure 8: Linear relationship between bacterial activity (BQV/mL) and total suspended
790 solid concentration (mg/L) for control (open symbol) and treatment RAS (solid symbol) and
791 in sum. The dashed line shows the overall linear relationship, the solid lines show the
792 linear relationship of the treatment and control system respectively.

793

794 Highlights:

- 795 • Study of combined chronic effects of critical solid and unionized ammonia exposure
- 796 • Full control of water parameters except turbidity in replicated RAS
- 797 • Only minimal effects of $\text{NH}_3\text{-N}$ up to 0.05 mg/L on fish physiology
- 798 • No interaction effects between ammonia and suspended solid load
- 799 • Close linear correlation of suspended solid load and bacterial activity

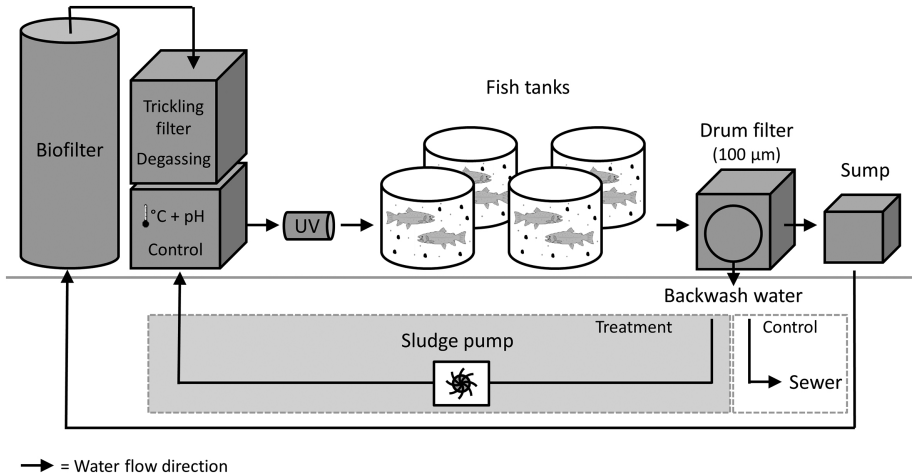


Figure 1

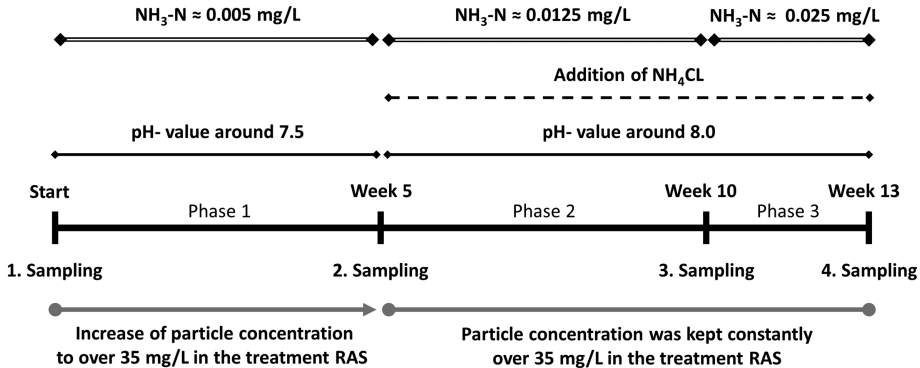


Figure 2

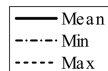
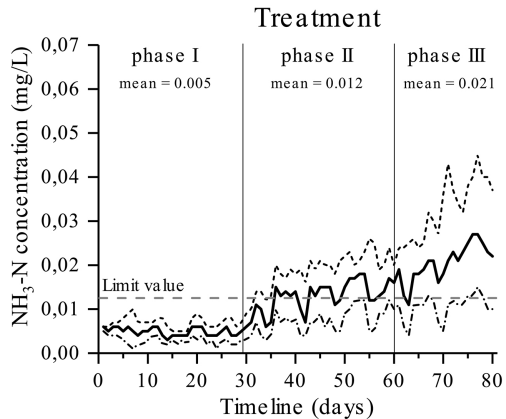
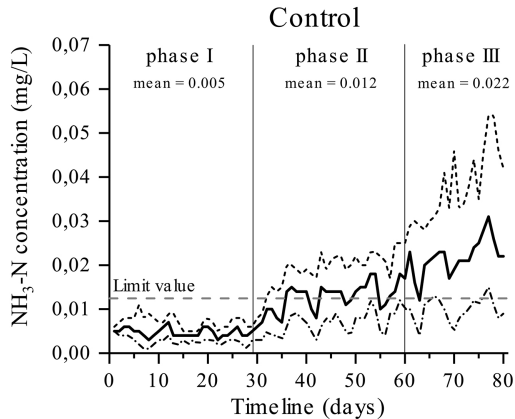


Figure 3

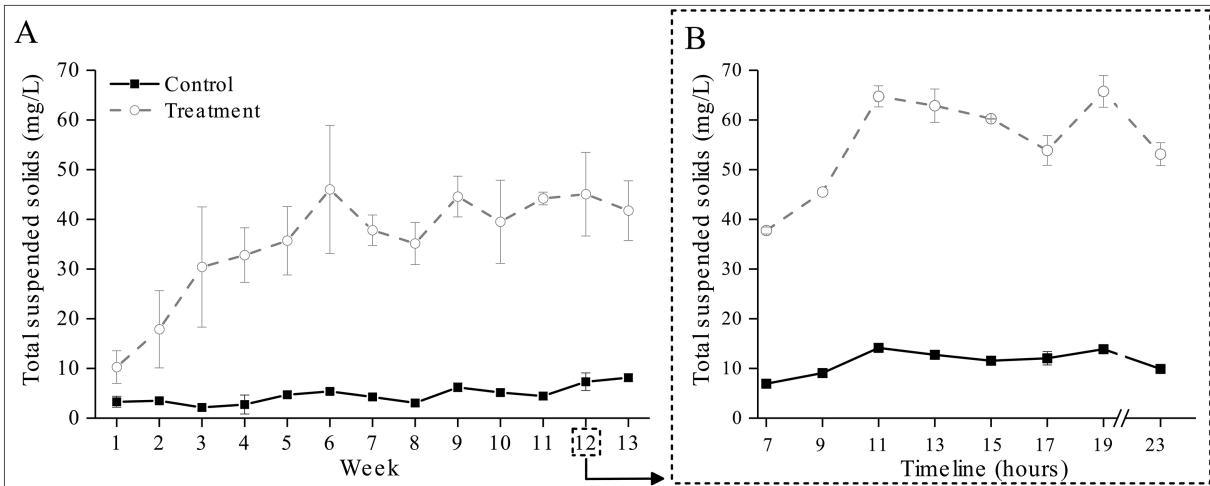


Figure 4

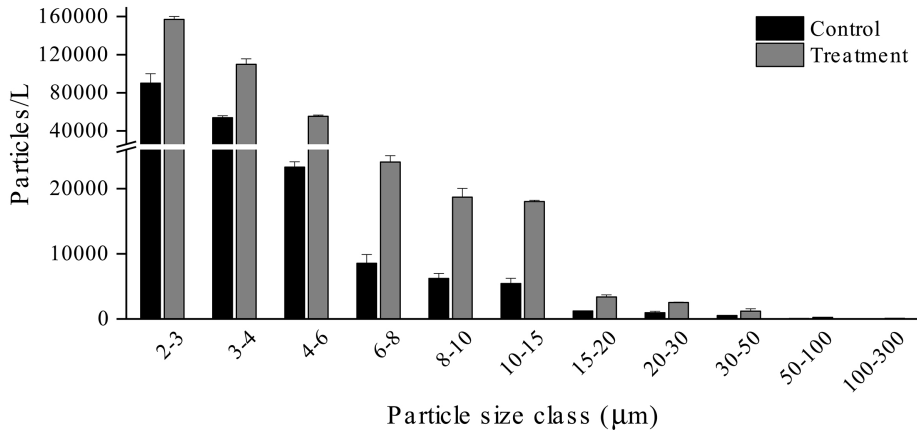
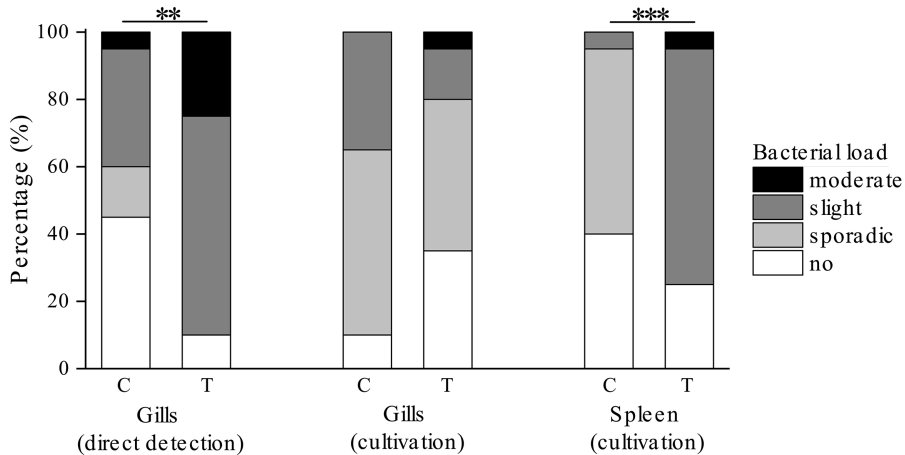


Figure 5



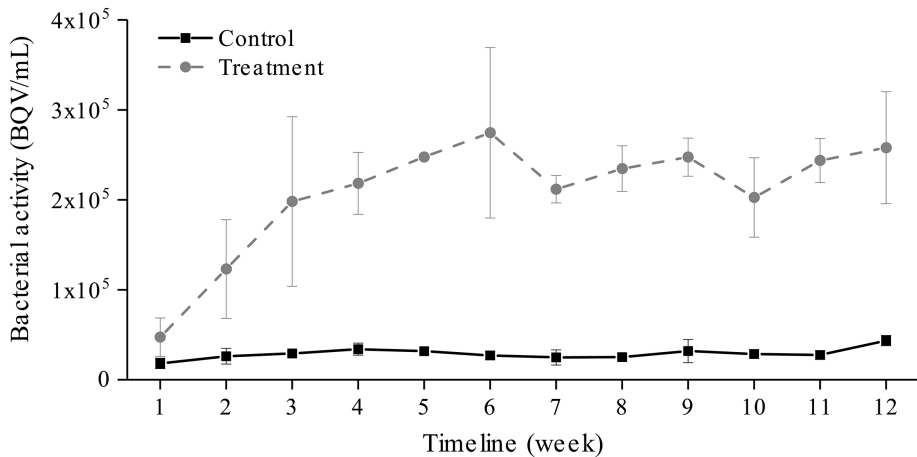


Figure 7

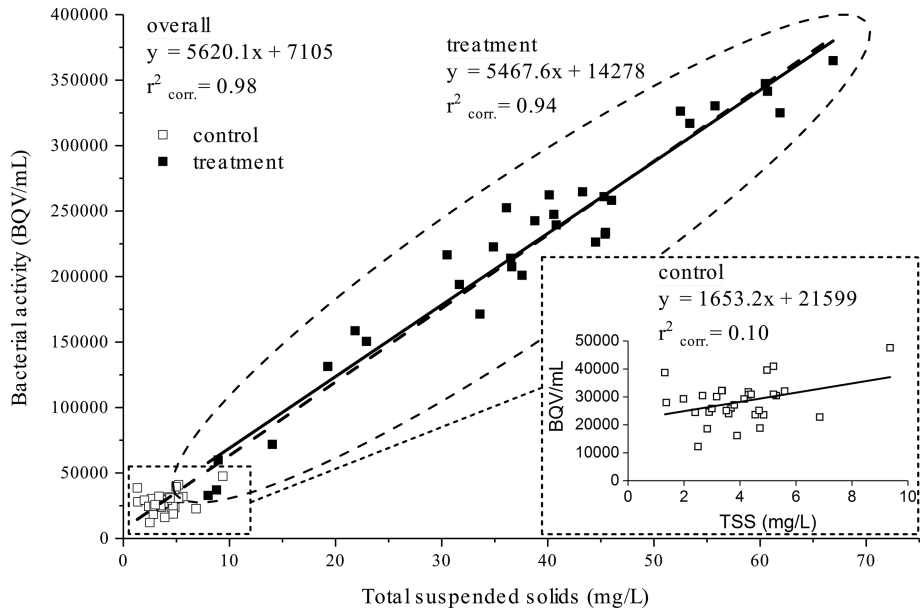


Figure 8